Staining Treponema Pallidum And Other Treponemata

By B. S. LEVINE, Ph.D.

The principles of staining necessary to obtain suitable results in the direct microscopic count of milk have been outlined in a previous publication (*1*). That study was originally undertaken to improve milk-film staining procedures. However, the principles discussed apply equally to other types of biological staining, and study has been made of the staining of spirochetes, especially those of the *pallidum* type.

A comprehensive review of the literature pertaining to the staining of *Treponema pallidum* was presented by Campbell and Rosahn (*2*), and recently DeLamater and others (*3*) have described a new modification of the Fontana staining procedure. Campbell and Rosahn classify all previously recommended procedures for staining *T. pallidum* into two groups. In the first group, the spirochetes are impregnated by a dye or a metallic ion and made visible against a pale background. In the procedures of the second group, the background is darkened by a material such as India ink or by an alteration in the method of illumination, as in the dark-field procedure. The value of the dark-field method of examination in the hands of an experienced person can hardly be overestimated as a rapid and reliable diagnostic aid. However, efforts to develop a quick and easy staining procedure for the demonstration of spirochetes, and especially of *T. pallidum*, have never abated.

Concerning the impregnation of spirochetes by dyes and metallic ions, the statements made by Campbell and Rosahn can well be repeated. These authors state: "At one time or another practically all the dyes utilized by the histologist have been employed in efforts to stain the spirochete. In all cases simple aniline dyes alone have not succeeded in staining the organism sharply, and only when a suitable mordant was employed was the stain at all reliable." Most staining procedures based on the mordanting principles are complex, time consuming, inconsistent in the hands of the same technician, and frequently result in complete failure in the hands of well-trained laboratory workers. Silver impregnation techniques, while apparently highly specific for spirochetes, are not adaptable to routine laboratory work. They appear to be best suited for tissues. In addition, as aptly stated by Campbell and Rosahn, "Silver impregnation techniques when applied to smears have for the most part resulted in atypical forms with marked changes in the regularity and shape of the spirals. . . ."

Dr. Levine is a bacteriologist with the research and development branch of the Public Health Service Environmental Health Center in Cincinnati. *This paper was presented at the general session of the meeting of the Society of American Bacteriologists on May 31, 1951, at Chicago, Ill.*

The Staining Procedure

Survey of the literature cited, personal interviews with research workers in this field, and visits to several venereal disease research labor-

atories and syphilis diagnostic clinics indicated the need for the development of a spirochetal staining procedure which could be used routinely in clinical and public health laboratories. Based upon experience with many spirochetal staining formulas and the principles of biologics staining cited (*1*), a number of procedures for the preparation of spirochetal slide speci-

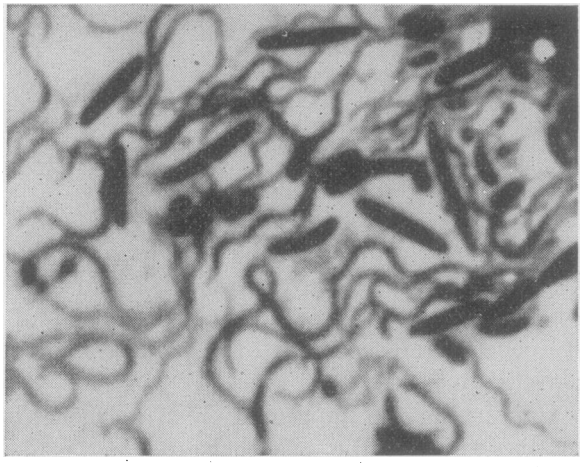

Figure 1. Trench mouth smear (×3,300).

mens and a number of staining solutions considered appropriate were prepared. Staining tests made led to the development of the following staining procedure.

Preparation of Smears or Films

The materials tested were: (1) culture suspensions of the following avirulent spirochetes—(*a*) Reiter's, (*b*) Nichols, and (*c*) the Kazan strain; (2) oral smears from persons known to harbor a variety of spirochetes; (3) suspensions of testicular material of rabbits experimentally infected with Nichols strain of *T. pallidum;* (4) suspension of testicular material of a rabbit experimentally infected with a strain of *Treponema cuniculi;* and (5) smears from clinical cases having genital lesions which were positive by dark-field examination. In all cases the material was spread thinly over an area approximately 1 cm. square, and dried in the air. The slides were then placed in a removable slide tray or a Coplin jar and defatted for 2 minutes or longer with U.S.P. chloroform, made acid-free with an excess of sodium or potassium carbonate. The slides were then drained and dried free from chloroform. They were again submerged for 2 minutes or longer into another glass container filled with 95-percent ethanol or methanol likewise made acid-free by adding an excess of sodium or potassium carbonate. The slides were again drained and dried free of the alcohol.

Preparation of Stock and Final Stain Solutions

Staining of any of the previously mentioned spirochete-containing materials can be accomplished either with crystal violet or with basic fuchsin. The use of certified dyes is recommended. Prepare: (1) a 2.5-percent solution of sodium or potassium carbonate in distilled water and (2) a 1-percent solution of crystal violet or of basic fuchsin in distilled water. These should be labeled "Stock Solutions." They can be kept indefinitely without serious deterioration.

For the preparation of the final staining solution, place 89 ml. of distilled water in a clean glass beaker. Add to this 1 ml. of either the potassium or sodium carbonate stock solution. Mix well. To this add rapidly 10 ml. of either

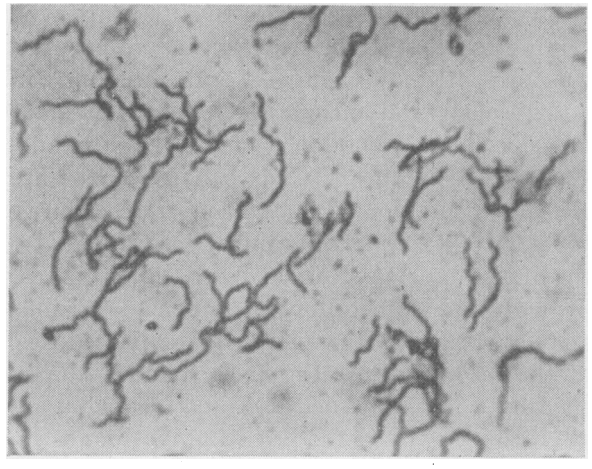

Figure 2. Culture of avirulent Reiter's spirochete (×1,500).

crystal violet or basic fuchsin stock dye solution. Mix well. The final stain-solution should not be prepared until the slides are ready for staining. Staining should be done as soon as possible after the final stain-solution has been prepared. It is recommended that after 4 to 6 hours a new final stain-solution be made. The addition to this final staining solution of sodium bicarbonate in varying amounts tends to stabilize it, so that it can be used for a longer

period of time. Such stabilization of the final staining solution as indicated thus far appears to add nothing to the spirochete-staining properties of the solution.

Staining of Slide Specimens

Place the previously prepared slide specimens in a Coplin jar or removable slide tray and MacCallum type of staining dish. Fill the staining dish with the final crystal violet or basic fuchsin stain-solution until slides are completely submerged. Two minutes is a sufficient time interval for proper staining. Leaving the slide specimens in the final stain-solution for a longer period will not cause overstaining. It is, however, considered best to adhere to the 2-minute staining interval. Remove the slides from the staining solution one at a time or, if a tray is used, remove the entire lot and rinse lightly in a beaker or other suitable glass dish containing tap water until it is judged that excess stain has been removed. Drain, air dry, and examine microscopically.

It was observed on numerous occasions that the presence of phosphates in the preparation material appears to interfere with the proper staining of the finer forms of the spirochetes. It is therefore suggested that extraction of testicular material be made with distilled water or with isotonic saline.

Under the microscope, treponemata, especially *T. pallidum*, stained with basic fuchsin appear to be very slender and to have a smooth and even surface. Crystal violet imparts to the spirochetes a greater thickness and a certain coarseness of surface. With either stain, however, the morphology of the spirochetes remains unaffected. For this reason it is rather easy, after some experience, to differentiate *T. pallidum* as it occurs in rabbit testicular or clinical material from the avirulent types as presented by the cultures previously mentioned. Differentiation is not so simple with oral smears, for in these, on occasion, spirochetes which only a few microscopists can differentiate with any degree of certainty are observed. Clinical syphilologists say that in the case of suspicious genital lesions the proper decision can be arrived at with ease, as it is generally believed that spirochetes other than *T. pallidum* do not invade the deeper tissues.

Precautions

Attention is invited to the following consideration: The standing procedure described takes into account certain principles of adsorption. Each step outlined is designed to meet certain specific conditions of such adsorption. It is therefore recommended that the procedure be adhered to as closely as possible. It is especially recommended that the prepared slide specimen be submerged into the final dye solution edgewise, as previously described. Placing the slide specimen horizontally on a staining table and flooding it with the final dye solution is definitely not recommended.

Advantages of the New Staining Procedure

The advantages of the staining procedure described are as follows: No mordants are required; no heating of the specimens or of any of the solutions is necessary; the reagents used are commonly found in any public health or clinical laboratory; staining can be accomplished within 5 to 10 minutes; a trayful of slide specimens takes no longer to stain than a single specimen; the tolerance range with regard to the prevailing pH of any of the solutions is rather wide, thus eliminating the need for unusual care in the staining process; the slides can be destained and restained any number of times (as will be described later) without in any way affecting the original staining properties or the morphology of the spirochetes. Permanent mounts can be made or the specimens can be destained and restained if fading has occurred, or, if it is desired, the same slide may be studied as stained first with the crystal violet and then with the basic fuchsin, or conversely. Other advantages of this staining procedure may make themselves evident to the laboratory worker as he gains experience with it. What has been specifically mentioned is sufficient to indicate that the staining procedure described appears adaptable to routine laboratory procedure.

To destain and restain a specimen, treat the slide as if it had not been stained. First, defat in the acid-free chloroform; this will remove the immersion oil and will almost completely destain the specimen. Second, submerge the

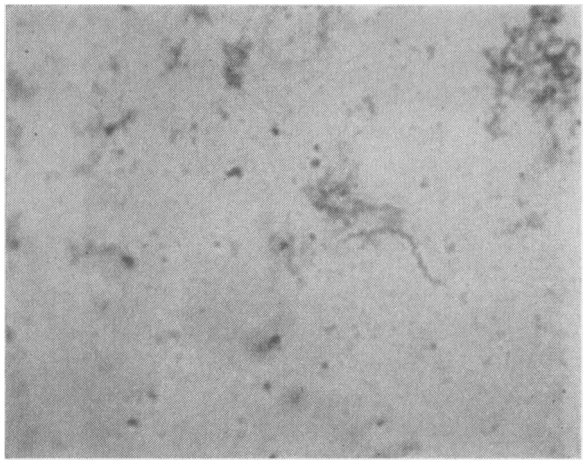

Figure 3. From experimental rabbit syphilis; Nichols virulent strain (×1,500).

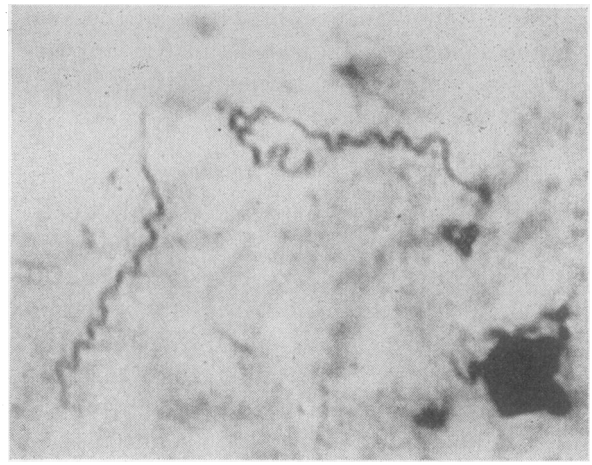

Figure 4. From primary penile lesion Alto Medical Center, Alto, Ga. (×3,300).

slide in acid-free ethanol or methanol. This will complete the destaining of the slide. Restain as described. For classroom demonstration or exercise, and where securing of new slide specimens presents difficulties, a set of slides once used can be destained and presented to new students as if the slides had not been previously used. It is, of course, necessary to take care that the material forming the film is not destroyed by scratching it off in part or in toto through careless handling.

Because of the simplicity of this spirochetal staining procedure and the reproducibility of results even upon destaining and restaining, it can be used in determining the rate of spirochetal growth in cultures by standardizing microscopic count procedures similar to the direct microscopic count of milk (*4*). Slides thus prepared can be filed for record purposes or for simultaneous comparative studies. In experimental rabbit syphilis, testicular material can be aspirated at certain intervals, and studies made microscopically of the degree of spirochetal proliferation, of the maturity of the treponemata, of the presence of involutional or otherwise unusual forms, as well as of the presence of possible contaminants.

Use in Epidemiological Studies

It has been suggested by some public health syphilologists that the staining procedure here described might find practical and useful application in the field of syphilis epidemiology. For example, it has been known for some time that a patient with a genital lesion of a suspicious type usually reports to the nearest general practitioner for examination. Such practitioners, as a rule, are not equipped to make dark-field examinations in order to ascertain the true nature of the lesion. As a matter of public health safety the assumption is usually made that the lesion is a specific one, and the patient is made noninfectious by an adequate administration of penicillin. The patient is then directed to report to a public health clinic. However, by the time the examining physician in the public health clinic sees such patients the primary infective agents causing the chancre have been eliminated by the penicillin. The proper epidemiological study of such cases thereby has been complicated or rendered impossible.

Using the spirochetal staining procedure described, situations such as have been mentioned can be handled as follows: (1) Physicians can be instructed to make slide smears of suspected lesions of their patients prior to administration of the penicillin; (2) the slides, properly labeled for identification, can then be sent to the public health laboratory for staining and microscopic examination; and (3) the epidemiological records can thereby be made complete.

Summary

Staining procedures offered heretofore for the examination of materials suspected of har-

boring virulent and avirulent spirochetes have been briefly reviewed and evaluated. A new, simple staining procedure for similar purposes is described. It appears suitable for routine laboratory use, in classroom work, and in research studies. However, no attempt should be made to employ this staining procedure in the place of the dark-field examination or any other established diagnostic procedure until sufficient evidence accumulated on a broad basis indicates that this staining procedure is dependable.

ACKNOWLEDGMENTS

The author acknowledges the cooperation given him by authorities of the Venereal Disease Research Laboratory, the officer in charge of the Alto Medical Center, Alto, Ga., and the photographic section of the Communicable Disease Center, all of the Public Health Service; and of Dr. J. H. Stokes, Dr. Harry E. Morton, and Noel Rose, of the Institute for the Study of Venereal Diseases of the University of Pennsylvania.

REFERENCES

(*1*) Levine, B. S.: Improving staining procedures in the direct miscroscopic examination of milk. J. Milk & Food Tech. 13: 321–329 (1950).

(*2*) Campbell, R. E., Rosahn, P. D.: The morphology and staining characteristics of *Treponema pallidum*. Review of the literature and description of a new technique for staining the organism in tisues. Yale J. Biol. & Med., 22: 527–543 (1950).

(*3*) DeLamater, E. D., Hannes, M., and Wiggall, R. H.: Studies on the life cycle of spirochetes. II. The development of a new stain. Am. J. Syph., Gonor. & Ven. Dis., 34: 515–518 (1950).

(*4*) American Public Health Association: Standard methods for the examination of dairy products: Microbiological, bioassay and chemical. New York, 1948, pp. 107–117.

Extension Service in Parasitology

A new extension service program in parasitology is being developed by the laboratory branch of the Communicable Disease Center, Public Health Service, Atlanta, Ga. The program will be available only to students who have completed the CDC parasitology courses in the laboratory diagnosis of intestinal or blood parasites, beginning with the 1951 fall group of graduates. For a specified time following completion of the laboratory course, the students will receive specimens for examination and identification.

This program replaces the service which was in operation by the CDC parasitology laboratory from 1945 to January 1, 1951. This service was terminated partly because the list of recipients had grown beyond the facilities for collection and preparation of materials for this purpose.

Students who completed the parasitology courses earlier than the fall of 1951 may request parasitology loan sets at any time.

An extension service program covering all specialties is being contemplated for the State public health laboratories. Details of the program will be submitted to the State health laboratory directors in the near future for their consideration and comment.